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(54) Title: PROMOTING CELL GROWTH BY INHIBITING ENABLED PROTEINS OR THEIR LIGANDS

(57) Abstract: The invention provides methods for modulating cell growth by altering the binding of Ena/VASP proteins with their ligands. Disclosed is a method of promoting growth or movement of a cell comprising (i) an Ena/VASP protein comprising an Enabled VASP homology 1 (EVH1) domain and (ii) a proline rich EVH1 peptide (PREP) ligand of said Ena/VASP protein wherein the cell is subject to growth or movement inhibition mediated by the binding of the EVH1 domain to the PREP ligand. The method involves specifically and artificially inhibiting the binding of the EVH1 domain to the PREP ligand and confirming a resultant promotion of growth or movement of the cell. Converse methods are used to inhibit growth or movement of a cell.

Promoting Cell Growth by Inhibiting Enabled Proteins or Their Ligands

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INTRODUCTION

Field of the Invention

The field of the invention is regulating cell growth by inhibiting Enabled proteins binding to their ligands.

Background of the Invention

The Enabled VASP homology 1 (EVH1) domain is an interaction module found in several proteins implicated in cell growth and motility (e.g. Prehoda et al. 1999, Cell 97, 471-480). EVH1 domains bind a proline rich EVH1 peptide (PREP), such as found in Robo proteins (e.g. Kidd et al. 1998a, Cell 92, 205-215; Kidd et al. 1998b, Neuron 20, 25-33; Kidd et al. 1999, Cell 96, 785-794) Interactions between EVH1 proteins and PREP ligands are widely believed to promote cell growth and motility (e.g. Rottner et al. 1999, Nat Cell Biol 1, 321-322; Lanier et al. 1999, Neuron, 22, 313-325). In contrast, the present invention provides for promoting cell growth by inhibiting interactions between EVH1 proteins and PREP ligands.

SUMMARY OF THE INVENTION

The invention provides methods for modulating cell growth by altering the binding of Ena/VASP proteins with their ligands. In a particular embodiment, the invention provides a method of promoting growth or movement of a cell, said cell comprising (i) an Ena/VASP protein comprising an Enabled VASP homology 1 (EVH1) domain and (ii) a proline rich EVH1 peptide (PREP) ligand of said Ena/VASP protein wherein the cell is subject to growth or movement inhibition mediated by the binding of the EVH1 domain to the PREP ligand, the method comprising the steps of: specifically and artificially inhibiting the binding of the

EVH1 domain to the PREP ligand, detecting a resultant inhibition of the binding of the EVH1 domain to the PREP ligand and confirming a resultant promotion of growth or movement of the cell. Converse methods are used to inhibit growth or movement of a cell.

In more particular embodiments, the PREP ligand comprises a LPPPP Robo1-type peptide, which may be part of a Robo protein; the cell is a neuron, which may be in situ or in vitro; the cell, or a progenitor of the cell, is artificially transformed to express the Ena/VASP protein or the PREP ligand; the inhibiting step comprises introducing into the cell an antagonist which inhibits the binding of the EVH1 domain to the PREP ligand; or the inhibiting step comprises introducing into the cell a polynucleotide which inhibits expression of the Ena/VASP protein or the PREP ligand, thereby reducing the binding of the EVH1 domain to the PREP ligand.

DETAILED DESCRIPTION OF SPECIFIC EMBODIMENTS OF THE INVENTION

The cells used in the subject methods comprise (i) an Ena/VASP protein comprising an Enabled VASP homology 1 (EVH1) domain and (ii) a proline rich EVH1 peptide (PREP) ligand of said Ena/VASP protein; and are subject to growth or movement inhibition mediated by the binding of the EVH1 domain to the PREP ligand. Ena/VASP proteins and their ligands are well-known and defined in the art; for example, PREP ligands are natural Ena/VASP peptide ligands sharing a proline-rich consensus sequence (see e.g. Prehoda et al, supra). A wide variety of animal cells, especially mammalian cells, naturally express Ena/VASP proteins and their ligands, including neurons, epithelial cells, fibroblasts, endothelial cells, immune cells such as lymphocytes, etc. In addition, cells may be genetically modified by standard methods to express Ena/VASP proteins or PREP ligands (see, e.g. Molecular Cloning, A Laboratory Manual (Sambrook, *et al.* Cold Spring Harbor Laboratory), Current Protocols in Molecular Biology (Eds. Ausubel, *et al.*, Greene Publ. Assoc., Wiley-Interscience, NY) or that are otherwise known in the art).

Any convenient method may be used to specifically and artificially inhibit (or enhance) the binding of the EVH1 domain to the PREP ligand, detect a resultant inhibition of the binding of the EVH1 domain to the PREP ligand and confirm a resultant promotion (or inhibition) of growth or movement of the cell. For example, inhibition may be effected by introducing into the cell an antagonist which inhibits the binding of the EVH1 domain to the

PREP ligand. Such antagonists encompass competitive binding peptides (e.g. Prehoda et al., supra; Southwick and Purich, 1994, Proc. Natl Acad Sci USA 91, 5168-5172, etc.); EVH1 domain or PREP ligand-specific antibodies or intrabodies (e.g. see, e.g Harlow and Lane (1988) Antibodies, A Laboratory Manual, Cold Spring Harbor Laboratory; vonMehren M,Weiner LM. (1996) *Current Opinion in Oncology*. 8: 493-498, Marasco WA. (1997) *Gene Therapy*. 4: 11-15, Rondon IJ,Marasco WA. (1997) *Annual Review of Microbiology*. 51: 257-283); inhibitors defined in in vitro binding assays (e.g. Examples I and II, below) or conventional cell- or animal-based cell growth or motility assays (e.g. Examples III and IV, below).

Similarly, a wide variety of assays are available for detecting EVH1 domain - PREP binding inhibition, such as the EVH1 domain - PREP binding and functional assays described or cited herein; and for confirming promotion (or inhibition) of cell growth or movement, such as cell based time-lapse videomicroscopy assays to detect changes in cell growth or movement; animal based assays for epithelial wound healing (e.g. Martin et al., Nature 1992 Nov 12;360(6400):179-83), nerve growth and regeneration (e.g. Chen et al., 2000 Nature 403, 434-439; GrandPre et al., 2000 Nature 403, 439-444) hair growth (e.g. Li et al., J Dermatol 1999 Apr;26(4):203-209), immune cell migration, etc.

In a particular application, the target cells are vertebrate neurons, such as injured mammalian neurons in situ, e.g. Schulz MK, et al., Exp Neurol. 1998 Feb; 149(2): 390-397; Guest JD, et al., J Neurosci Res. 1997 Dec 1; 50(5): 888-905; Schwab ME, et al., Spinal Cord. 1997 Jul; 35(7): 469-473; Tatagiba M, et al., Neurosurgery. 1997 Mar; 40(3): 541-546; and Examples III and IV, below. A wide variety of methods may be used to introduce the antagonist into such target cells. For example, for CNS administration, a variety of techniques is available for promoting transfer of therapeutic agents across the blood brain barrier including disruption by surgery or injection, drugs which transiently open adhesion contact between CNS vasculature endothelial cells, and compounds which facilitate translocation through such cells. The compositions may also be amenable to direct injection or infusion, intraocular administration, or within/on implants e.g. fibersllagen fibers, in osmotic pumps, grafts comprising appropriately transformed cells, etc. In a particular embodiment, the antagonist is delivered locally and its distribution is restricted. For example, a particular method of administration involves coating, embedding or derivatizing fibers, such

as collagen fibers, protein polymers, etc. with therapeutic agents, see also Otto et al. (1989) J Neuroscience Research 22, 83-91 and Otto and Unsicker (1990) J Neuroscience 10, 1912-1921. The amount of antagonist administered depends on the antagonist, formulation, route of administration, etc. and is generally empirically determined and variations will necessarily occur depending on the target, the host, and the route of administration, etc. The compounds may be advantageously used in conjunction with other neurogenic agents, neurotrophic factors, growth factors, anti-inflammatories, antibiotics etc.; and mixtures thereof, see e.g. *Goodman & Gilman's The Pharmacological Basis of Therapeutics*, 9th Ed., 1996, McGraw-Hill, esp. Chabner et al., *Antineoplastic Agents* at pp.1233.

Alternatively, the inhibition may be effected by introducing into the cell a polynucleotide which inhibits expression of the Ena/VASP protein or the PREP ligand, thereby reducing the binding of the EVH1 domain to the PREP ligand. Such genetic therapies may be effected at the level of transcripts (e.g. using antisense oligonucleotides to inhibit translation) or at the genomic level (e.g. using genomic knockout vectors or genetic blockers of transcription). Gene therapy technologies for practicing these methods are known in the art.

Analogous methods are used for specifically and artificially enhancing the binding of the EVH1 domain to the PREP ligand and confirming a resultant inhibition of growth or movement of the cell. In these embodiments, an agonist of binding is an agent which promotes and/or enhances binding of the EVH1 domain to the PREP ligand.

EXAMPLES

I. Far Western Protein Interaction Assay

PREP ligands are generated as fusions to glutathione S-transferase (GST) by ligating the appropriate oligonucleotides into the BamHI and EcoRI restriction sites of plasmid pGEX4T-1 (Pharmacia). GST fusion proteins are expressed in *E. coli* strain TG1 by growing a 1 I culture to OD_{600} 5 1 and inducing with 1 mM IPTG. After 3 hr of further growth, cells are harvested, lysed by sonication, and the fusion protein purified on 100 mL of glutathione agarose resin (Sigma). After washing with 25 mM Tris HCI (pH 7.5), 150 mM NaCl, 1 mM EDTA, the resin is boiled in SDS-PAGE loading buffer and an aliquot electrophoresed on a 10%-20% SDS-PAGE gel. Fusion protein bands are transferred to nitrocellulose by

electroblotting and blocked for 1 hr at 4 °C in 50 mM Tris (pH 7.5), 150 mM NaCl, 0.1% TWEEN (TBST) with 1% nonfat milk and 1% bovine serum albumin.

Biotinylated EVH1 and control SH3 domain probes are produced as fusions to a naturally biotinylated *E. coli* protein using the PIN-POINT system (Promega). The DNA fragments encoding the desired protein segments (Mena 1-112, hWASP 1-170, dHomer 1-112, mSrc 87-142) are amplified from human, mouse, or *Drosophila* cDNA libraries by PCT and ligated into the BamHI and NotI sites of the plasmid PINPOINT Xa-3. The PINPOINT fusion proteins are expressed as described for the GST fusion proteins. The sonicated and centrifuged lysate is used directly, after estimating the fusion protein concentration by SDS-PAGE.

To detect binding, blocked nitrocellulose filters bearing the ligand fusion proteins are incubated at 4°C for 45 min with the appropriate biotinylated EVH1 domain probe at a concentration of ~0.25 mg/ml in 5 ml of the above blocking buffer. Filters are washed three times with ~100 ml TBST, incubated for 10 min with four units of streptavidin-conjugated horseradish peroxidase (Boehringer Mannheim) in 20 ml TBS (TBST without TWEEN), washed three times in ~100 ml TBST, and the bands visualized by enhanced chemiluminescence.

II. Fluorescence Peptide Binding Assay

Fluorescence titration is used to quantify PREP ligand binding affinities to EVH1 domains. Increasing amounts of peptide, from a 50 mM peptide stock, are titrated into a stirred cuvette with a 1.25 ml solution of 0.5 mM Mena (residues 1-112) in 20 mM HEPES (ph 7.5), 20 mM NaCl at 20 °C. The intrinsic fluorescence (excitation 280nm, emission 330 nm) of the Mena EVH1 domain over the course of the titration is monitored with a PTI fluorometer. At saturation in the absence of inhibitor, peptide binding results in an ~1.2 fold increase in fluorescence emission intensity. Fluorescence signal at each point is averaged for at least 30 s. For each experiment, at least 12 concentration points are measured. Intensity measurements are corrected for dilution and the resulting binding isotherm fit using the program proFit (Quantum Soft) to determine dissociation constants (K_D).

Table 1. In Vitro Antagonist of EVH1 domain - PREP binding in Far Western Protein

Interaction and Fluorescence Peptide Binding Assays

Antagonist	Class/Source	Far Western Interaction	Fluorescence Peptide Binding
1. PI-TK347	natural peptide	++++	++++
2. PI-JG04994	synthetic peptide	++++	++++
3. PI-JG17764	synthetic peptide	++++	++++
4. NI-LM1133	natural extract library	++++	++++
5. NI-LN0808	natural extract library	++++	++++
6. SI-RQ4442	synthetic library	++++	++++
7. SI-RR6740	synthetic library	++++	++++
8. CI-AB18355	combinatorial library	++++	++++
9. CI-AB21492	combinatorial library	++++	++++
10. CI-AB33177	combinatorial library	++++	++++

III. Corticospinal Tract (CST) Regeneration Assay

Antagonist of EVH1 domain - PREP ligand binding improve corticospinal tract (CST) regeneration following thoracic spinal cord injury by promoting CST regeneration into human Schwann cell grafts in the methods of Guest et al. (supra). For these data, the human grafts are placed to span a midthoracic spinal cord transection in the adult nude rat, a xenograft tolerant strain. Antagonists (see Table 1) incorporated into a fibrin glue are placed in the same region. Anterograde tracing from the motor cortex using the dextran amine tracers, Fluororuby (FR) and biotinylated dextran amine (BDA), are performed. Thirty-five days after grafting, the CST response is evaluated qualitatively by looking for regenerated CST fibers in or beyond grafts and quantitatively by constructing camera lucida composites to determine the sprouting index (SI), the position of the maximum termination density (MTD) rostral to the GFAP-defined host/graft interface, and the longitudinal spread (LS) of bulbous end terminals. The latter two measures provide information about axonal die-back. In control animals (graft only), the CST do not enter the SC graft and undergo axonal die-back. As shown in Table 2, the antagonists dramatically reduce axonal die-back and cause sprouting.

Table 2. In Vivo Neuronal Regeneration with Exemplary Antagonist Formulations

Antagonist	Formulation	Reduced Die-Back	Promote Sprouting
1. PI-TK347	5 uM	++++	++++
2. PI-JG04994	5 uM	++++	++++
3. PI-JG17764	5 uM	++++	++++
4. NI-LM1133	100 uM	++++	++++
5. NI-LN0808	25 uM	++++	++++
6. SI-RQ4442	100 uM	++++	++++
7. SI-RR6740	50 uM	++++	++++
8. CI-AB18355	20 uM	++++	++++
9. CI-AB21492	50 uM	++++	++++
10. CI-AB33177	25 uM	++++	++++

IV. Peripheral Nerve Regeneration Assay

EVH1 domain - PREP ligand binding antagonist are incorporated in the implantable devices described in US Pat No. 5,656,605 and tested for the promotion of in vivo regeneration of peripheral nerves. Prior to surgery, 18 mm surgical-grade silicon rubber tubes (I.D. 1.5 mm) are prepared with or without guiding filaments (four 10-0 monofilament nylon) and filled with test compositions comprising the antagonists of Table 1. Experimental groups consist of: 1. Guiding tubes plus Biomatrix 1™ (Biomedical Technologies, Inc., Stoughton, Mass) ; 2. Guiding tubes plus Biomatrix plus filaments; 3-23. Guiding tubes plus Biomatrix 1™ plus antagonists 1-10 of Table 1 (supra).

The sciatic nerves of rats are sharply transected at mid-thigh and guide tubes containing the test substances with and without guiding filaments sutured over distances of approximately 2 mm to the end of the nerves. In each experiment, the other end of the guide tube is left open. This model simulates a severe nerve injury in which no contact with the distal end of the nerve is present. After four weeks, the distance of regeneration of axons within the guide tube is tested in the surviving animals using a functional pinch test. In this test, the guide tube is pinched with fine forceps to mechanically stimulate sensory axons.

Testing is initiated at the distal end of the guide tube and advanced proximally until muscular contractions are noted in the lightly anesthetized animal. The distance from the proximal nerve transection point is the parameter measured. For histological analysis, the guide tube containing the regenerated nerve is preserved with a fixative. Cross sections are prepared at a point approximately 7 mm from the transection site. The diameter of the regenerated nerve and the number of myelinated axons observable at this point are used as parameters for comparison.

Measurements of the distance of nerve regeneration document the therapeutic effect of antagonists 1-100. Similarly, plots of the diameter of the regenerated nerve measured at a distance of 7 mm into the guide tube as a function of the presence or absence of one or more antagonists of the device demonstrate a similar therapeutic effect of all 10 antagonists tested. No detectable nerve growth is measured at the point sampled in the guide tube with the matrix-forming material alone. The presence of guiding filaments plus the matrix-forming material (no antagonist) induces only very minimal regeneration at the 7 mm measurement point, whereas dramatic results, as assessed by the diameter of the regenerating nerve, are produced by the device which consisted of the guide tube, guiding filaments and antagonist compositions. Finally, treatments using guide tubes comprising either a matrix-forming material alone, or a matrix-forming material in the presence of guiding filaments, result in no measured growth of myelinated axons. In contrast, treatments using a device comprising guide tubes, guiding filaments, and matrix containing antagonist compositions consistently result in axon regeneration, with the measured number of axons being increased markedly by the presence of guiding filaments.

The foregoing descriptions of particular embodiments and examples are offered by way of illustration and not by way of limitation. All publications and patent applications cited in this specification and all references cited therein are herein incorporated by reference as if each individual publication or patent application or reference were specifically and individually indicated to be incorporated by reference. Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be readily apparent to those of ordinary skill in the art in light of the teachings of this invention that certain changes and modifications may be made thereto without departing from the spirit or scope of the appended claims.

WHAT IS CLAIMED IS:

1. A method of promoting growth or movement of a cell,

said cell comprising (i) an Ena/VASP protein comprising an Enabled VASP homology 1 (EVH1) domain and (ii) a proline rich EVH1 peptide (PREP) ligand of said Ena/VASP protein

wherein the cell is subject to growth or movement inhibition mediated by the binding of the EVH1 domain to the PREP ligand, the method comprising the steps of:

specifically and artificially inhibiting the binding of the EVH1 domain to the PREP ligand, detecting a resultant inhibition of the binding of the EVH1 domain to the PREP ligand and confirming a resultant promotion of growth or movement of the cell.

2. A method according to claim 1, wherein the PREP ligand comprises a LPPPP peptide.

3. A method according to claim 1, wherein the PREP ligand comprises a LPPPP peptide and the LPPPP peptide is part of a Robo protein.

4. A method according to claim 1, wherein the cell is selected from a natural neuron, fibroblast, epithelial cell and lymphocyte.

5. A method according to claim 1, wherein the cell, or a progenitor of the cell, is artificially transformed to express the Ena/VASP protein or the PREP ligand.

6. A method according to claim 1, wherein the cell is in situ.

7. A method according to claim 1, wherein the cell is in vitro.

8. A method according to claim 1, wherein the inhibiting step comprises introducing into the cell an antagonist which inhibits the binding of the EVH1 domain to the PREP ligand.

9. A method according to claim 1, wherein the inhibiting step comprises introducing into the cell a polynucleotide which inhibits expression of the ENA/VASP protein or the PREP

ligand, thereby reducing the binding of the EVH1 domain to the PREP ligand.

10. A method according to claim 1, wherein the PREP ligand comprises a LPPPP peptide, the LPPPP peptide is part of a Robo1 protein, the cell is a natural neuron in situ and the inhibiting step comprises introducing into the cell an antagonist which inhibits the binding of the EVH1 domain to the PREP ligand.

11. A method of inhibiting growth or movement of a cell,

said cell comprising (i) an Ena/VASP protein comprising an Enabled VASP homology 1 (EVH1) domain and (ii) a proline rich EVH1 peptide (PREP) ligand of said Ena/VASP protein,

wherein the cell is subject to growth or movement inhibition mediated by the binding of the EVH1 domain to the PREP ligand, the method comprising the steps of:

specifically and artificially enhancing the binding of the EVH1 domain to the PREP ligand, detecting a resultant enhancement of the binding of the EVH1 domain to the PREP ligand and confirming a resultant inhibition of growth or movement of the cell.

12. A method according to claim 11, wherein the PREP ligand comprises a LPPPP peptide.

13. A method according to claim 11, wherein the PREP ligand comprises a LPPPP peptide and the LPPPP peptide is part of a Robo protein.

14. A method according to claim 11, wherein the cell is selected from a natural neuron, fibroblast, epithelial cell and lymphocyte.

15. A method according to claim 11, wherein the cell, or a progenitor of the cell, is artificially transformed to express the Ena/VASP protein or the PREP ligand.

16. A method according to claim 11, wherein the cell is in situ.

17. A method according to claim 11, wherein the cell is in vitro.

18. A method according to claim 11, wherein the enhancing step comprises introducing into the cell an agonist which enhances the binding of the EVH1 domain to the PREP ligand.

19. A method according to claim 11, wherein the enhancing step comprises introducing into the cell a polynucleotide which enhances expression of the ENA/VASP protein or the PREP ligand, thereby enhancing the binding of the EVH1 domain to the PREP ligand.

20. A method according to claim 11, wherein the PREP ligand comprises a LPPPP peptide, the LPPPP peptide is part of a Robo1 protein, the cell is a natural neuron in situ and the inhibiting step comprises introducing into the cell an agonist which enhances the binding of the EVH1 domain to the PREP ligand.